A Multipotent Progenitor Domain Guides Pancreatic Organogenesis

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SUMMARY

The mammalian pancreas is constructed during embryogenesis by multipotent progenitors, the identity and function of which remain poorly understood. We performed genome-wide transcription factor expression analysis of the developing pancreas to identify gene expression domains that may represent distinct progenitor cell populations. Five discrete domains were discovered. Genetic lineage-tracing experiments demonstrate that one specific domain, located at the tip of the branching pancreatic tree, contains multipotent progenitors that produce exocrine, endocrine, and duct cells in vivo. These multipotent progenitors are Pdx1^{+}Ptf1a^{+}cMyc^{High}Cpa1^{+} and negative for differentiated lineage markers. The outgrowth of multipotent tip cells leaves behind differentiated progeny that form the trunk of the branches. These findings define a multipotent compartment within the developing pancreas and suggest a model of how branching is coordinated with cell type specification. In addition, this comprehensive analysis of >1,100 transcription factors identified genes that are likely to control critical decisions in pancreas development and disease.

INTRODUCTION

Mammalian organs contain millions of cells and many different cell types, all of which arise from a small number of embryonic cells, the multipotent progenitors. Despite their importance, rather little is known about when multipotent progenitors arise or how they guide organ construction. The present study is concerned with multipotent progenitors in the pancreas. We identify genetic markers for these cells and show where they are positioned and how their progeny give rise to the branching organ and its exocrine and endocrine components.

The pancreas first appears as two separate buds around Embryonic Day (E) 9.5 through evagination of the early gut endoderm. The buds grow and initiate branching at around E11.5 by sending finger-like epithelial protrusions into the surrounding mesenchyme. Successive rounds of branching and further growth result in a three-dimensional organ with a tree-like epithelial network surrounded by mesenchyme. The adult pancreas is composed of three major cell types—endocrine, exocrine, and duct cells—all arranged in a stereotypic manner (Jensen, 2004; Murtaugh and Melton, 2003).

In recent years, many studies of pancreas development have focused on understanding the specification of individual cell types, especially endocrine cells (Wilson et al., 2003). Comparatively less is known about the multipotent cells of the embryonic pancreas, although it is generally assumed that they are present in pancreatic buds. Cells in the nascent pancreatic buds have a similar epithelial morphology and stain broadly for *Pdx1* and *Ptf1a*, two transcription factors (TFs) required for pancreas development (Jonsson et al., 1994; Kawaguchi et al., 2002; Offield et al., 1996). Furthermore, lineage studies have shown that cells expressing Pdx1 at this stage give rise to all adult pancreatic cell types (Gu et al., 2002). However, it is not known whether all of the Pdx1^{+} cells, or only a subset of them, are multipotent. During the branching stage, the pancreas becomes more complex in both structure and cell-type composition, and there is currently no information as to whether or where multipotent progenitors exist at this stage. All molecular markers identified to date, including *Pdx1* and *Ptf1a*, are expressed in multiple cell types during the branching stage, and none can be used to distinguish multipotent cells from committed cell types (Hale et al., 2005; Krapp et al., 1996; Sander et al., 2000; Sussel et al., 1998). Indirect evidence, however, suggests that multipotent progenitors are present in the branching stage; for instance, it has been observed that *Ngn3*$^{+}$ endocrine progenitors are continuously produced throughout embryogenesis from *Ngn3*$^{-}$ cells (Maestro et al., 2003). Although these *Ngn3*$^{-}$ cells may simply represent an earlier endocrine progenitor, they could also represent multipotent cells.

If multipotent pancreatic cells exist, where are they located and how do they construct the branching structures

of the pancreas? In the central nervous system, multipotent cells reside within a specific domain, the ventricular zone, with differentiated progeny migrating outward and organizing into different structures (Jessell, 2000). Similarly, in the gut, stem cells are localized at the base of the villi, sending differentiated progeny toward the tips (Fuchs et al., 2004). It is conceivable that a similar "multipotent domain" exists in the developing pancreas and that it serves as an organizing center for pancreatic organogenesis.

To identify previously unappreciated compartments in the pancreas, we performed genome-wide expression analysis of the developing pancreas with >1,100 TFs. Several discrete domains were recognized, representing the mesenchyme, epithelium, and vasculature of the developing pancreas. The epithelium domain can be further divided into two separate domains delineating the distal tip and trunk of the branches. Molecular marker analysis and genetic lineage tracing experiments suggest that cells of the distal tip domain, which coexpress Pdx1, Ptf1a, c-Myc, and Carboxypeptidase A1 (Cpa1), are multipotent progenitors that are capable of generating all pancreatic cell types, including exocrine, endocrine, and duct cells. The outgrowth of tip progenitors leaves behind endocrine and duct progeny that comprise the trunk of the branches. The multipotent tip progenitors undergo a developmental switch around midgestation (E14) and become exocrine cells. Our findings reveal the existence of a distinct domain of multipotent progenitors in the developing pancreas and, combined with earlier studies, suggest a model of how pancreatic branching is coordinated with cell-type specification.

RESULTS

Genome-Wide TF Expression Analysis Defines Five Domains of Gene Expression within the Developing Pancreas

A genome-scale, whole-mount in situ hybridization screen was performed with a recently developed mouse TF library that covers about 80% (~1,100) of all predicted mouse TFs (total, ~1,400) (Gray et al., 2004). E9.5 embryos and E14.5 dorsal pancreata were chosen for the screen. E9.5 represents the earliest stage of pancreatic organogenesis and E14.5 represents a stage at which the developing pancreas is undergoing active growth, branching, and cellular differentiation (Murtaugh and Melton, 2003).

Of the 1,100 TF genes analyzed, we detected the expression of 8 TF genes at E9.5 (see Figure S1 in the Supplemental Data available with this article online) and 94 TF genes at E14.5 (Table S1). The eight TF genes expressed at E9.5 are also present in E14.5 pancreas (Figure S1 and S2). Among TFs identified in the screen are many well-studied pancreatic genes, including *Pdx1*, *Ptf1a*, *Nkx2.2*, *and Ngn3* (reviewed by Edlund, 2002 and Wilson et al., 2003). In addition, we identified genes, the expression of which, in the pancreas, has not been described previously, including *Lisch7*, *Wbscr14*, and *Tbx3* (Table S1).

The whole-mount in situ samples provide a clear view of overall expression patterns, but lack cellular resolution for structures within the pancreas. In situ hybridizations on tissue sections were performed with all 94 TFs identified from the initial whole-mount screen (Figures 1F–1J). Of the 94 genes, 60 yielded a clear, regionalized signal at E14.5, and these could be classified into five general patterns (Figures 1K–1O and Table S1). For simplicity, we referred to them as "pan-epithelium," "tip," "trunk," "mesenchyme," and "vascular." Pan-epithelium genes, such as *Hex*, are expressed specifically in pancreatic epithelial but not mesenchymal cells (Figures 1A, 1F, and 1K). Tip genes are confined to the distal tips of the pancreatic epithelial tree (Figures 1B, 1G, and 1L, arrowheads), while trunk genes appear in cells scattered within the trunk but not the tips of the branching epithelium (Figures 1C, 1H, and 1M). In addition to these three epithelial domains, the mesenchyme and vascular classes of TFs have mesenchymal- and vascular-specific expression patterns, respectively (Figures 1D, 1E, 1I, 1J, 1N, and 1O).

Different Pancreatic Precursors Are Localized in Discrete Epithelial Domains

Given that all three major pancreatic cell types, including exocrine, endocrine, and duct cells, derive from the epithelium (Gu et al., 2002), we used specific markers to analyze whether the tip and trunk epithelial domains contain separate precursor populations.

Several tip genes, including *Ptf1a*, *Mist1*, and *RBP-J* (Table S1), have been implicated in the development of exocrine tissues (Beres et al., 2006; Lin et al., 2004; Pin et al., 2001; Zecchin et al., 2004). We tested the exocrine nature of the tip domain at E14.5 by double-labeled in situ hybridization of selected tip genes with *Carboxypeptidase A1* (*Cpa1*) and *Amylase*, two markers of fully differentiated exocrine cells. Complete overlap was observed (Figures 2A and 2B), suggesting that the tip domain is occupied by exocrine precursors at E14.5. These data are consistent with published results (Pictet and Rutter, 1972).

In contrast to tip genes, most trunk TFs identified are well-established markers of endocrine precursors, with the exception of vitamin D receptor (*VDR*) and *Wbscr14*, the expression of which in the embryonic pancreas had not been described before (Table S1). Double-labeled in situ hybridization revealed that *VDR* and *Wbscr14* partially overlap with a number of endocrine genes (not shown), suggesting that they are also expressed in endocrine cells. To visualize the extent of the pancreatic trunk that is occupied by endocrine precursors, a mixture of cRNA probes, including most of the endocrine precursor markers (*Ngn3*, *NeuroD*, *Pax4*, *Pax6*, *Isl1*, *Brn4*, *Myt1*, *MafB*, *Arx*, *Wbscr14*, and *VDR*) was used for in situ hybridization on E14.5 pancreas (Figure 2C). While the majority of trunk cells were labeled (Figure 2C), some appear to have been negative (Figure 2C, arrows). It is noteworthy that the expression of endocrine genes did not extend inside the tips (Figure 2C, arrowheads). These data indicate that endocrine precursor cells reside exclusively within the

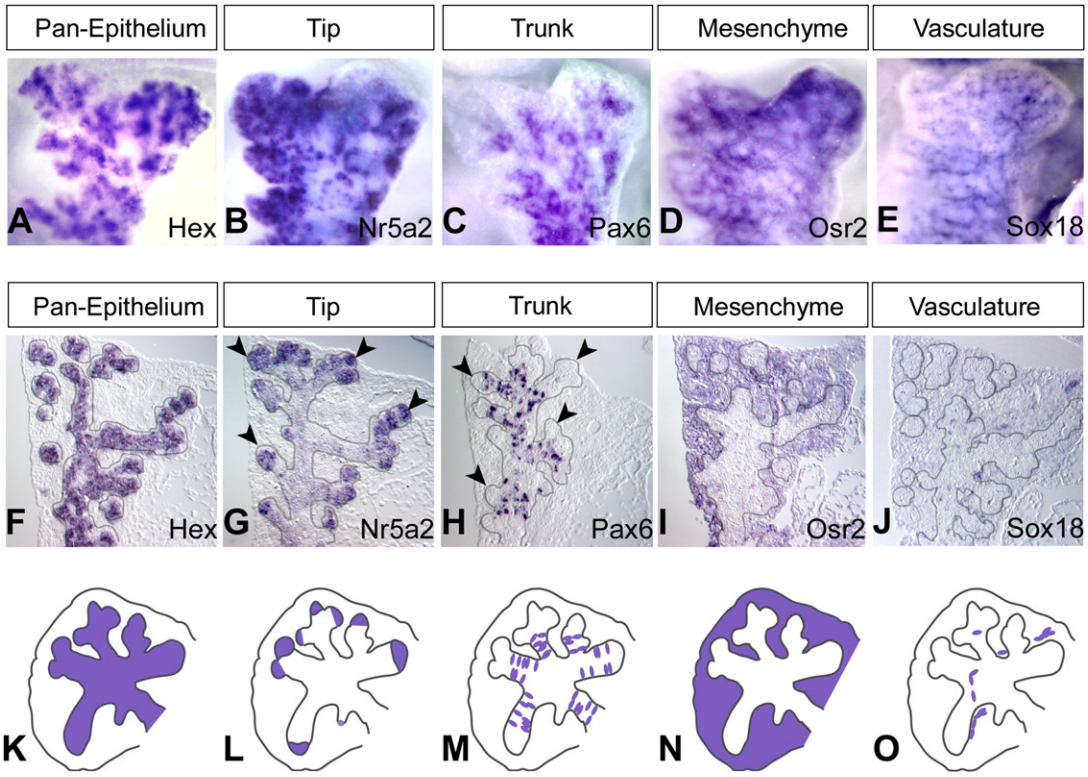

Figure 1. Genome-Wide TF Expression Analysis of the Developing Pancreas

(A–E) Whole-mount in situ hybridization screen was performed with ~1,100 mouse TFs on E14.5 dorsal pancreas. A total of 94 genes showed specific expression that can be classified into five broad patterns, as represented by the expression of *Hex*, *Nr5a2*, *Pax6*, *Osr2*, and *Sox18*, respectively.

(F–J) Corresponding section in situ hybridization images of the five patterns. Arrowheads in (G) and (H) indicate tips of the branching pancreatic tree. Pancreatic epithelia are outlined.

(K–O) Schematic drawings of pancreatic gene expression patterns.

trunk of the pancreatic branches. Due to lack of definitive precursor markers for the pancreatic duct lineage, we could not assess where duct precursors are localized within the branching structure.

To further confirm that the tip and the trunk of the pancreatic branches at E14.5 represent discrete domains, we performed double-labeled in situ hybridization of *Cpa1* (tip) and the endocrine progenitor marker *Ngn3* (trunk) (Gradwohl et al., 2000; Gu et al., 2002). No overlap was observed (Figure 2D). Together, our analysis suggests that the tip and trunk of the branching pancreatic tree at E14.5 are separate domains and contain different precursor populations.

We further analyzed the expression of tip genes at E12.5, when pancreatic branching morphogenesis has just begun. Although most tip genes yielded no staining at this early stage, signals were detected for *C-myc*, *Ptf1a*, and *Cpa1* in the newly formed branches (Figures 2E–2G, arrowheads). Interestingly, when one tip divides into two or more tips, the tip markers, Cpa1, Ptf1a, and c-Myc, are downregulated in the cleft region before overt morphological changes occur (Figure 2G, hollow arrowheads, and Figures S5A and S5B). Double-labeled in situ hybridization of Ptf1a, c-Myc, and Cpa1 shows that they are coexpressed in the same population of tip cells at E12.5 (Figures S5A and S5B). In addition, $Ngn3^+$ endocrine cells and $Cpa1^+$ tip cells occupy distinct domains (Figure 2H). Unlike at E14.5, however, the tip cells at E12.5 do not express the exocrine markers *Amylase* and *Elastase* (data not shown). These data raised the question of whether the $Ptf1a^+cMyc^+Cpa1^+$ tip cells that exist before E14.5 are multipotent progenitors or committed exocrine precursors.

Distal Tip Cells Represent a Novel, Fast-Proliferating Cell Type

Among the three early tip genes, *c-Myc* expression, though concentrated in the tips, is also detected at lower levels in other epithelial cells (data not shown). *Ptf1a* expression is rather weak before E12.5, and cannot be easily or reliably detected by antibody staining or in situ hybridization. In contrast, *Cpa1* expression is specific to the tip cells and readily detectable by antibody staining and in situ hybridization. We therefore used *Cpa1* as a marker to further characterize the tip cells during early pancreatic branching morphogenesis.

Cpa1 mRNA is first detected in E9.5 and protein expression starts around E10.5 in the pancreatic buds (data not shown). At E11.5, Cpa1 is expressed in a scattered population of epithelial cells (Figures 3A, 3E, and 3I). Shortly

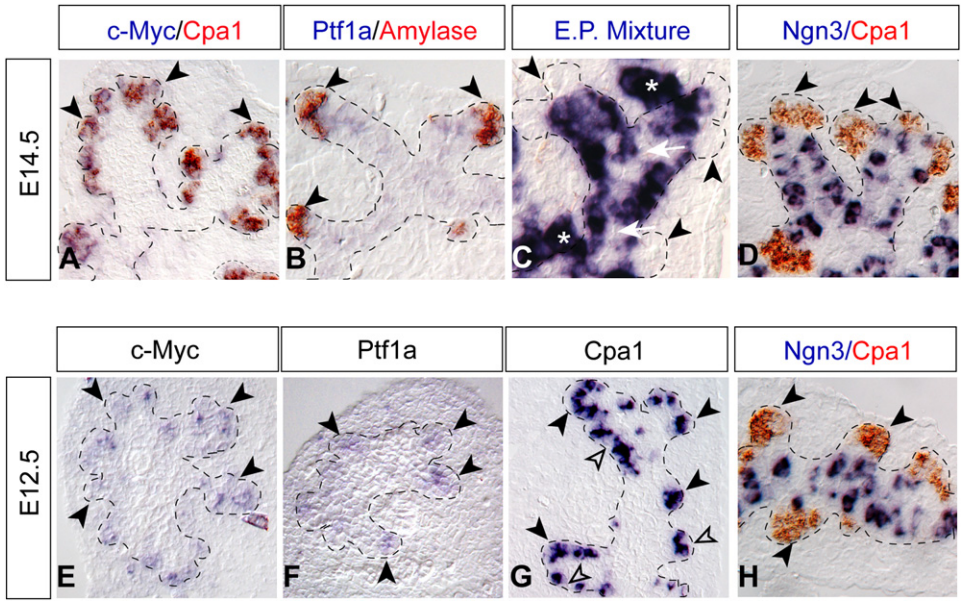

Figure 2. The Tip and Trunk of Epithelial Branches Contain Different Precursor Cells

(A and B) At E14.5, tip markers *c-Myc* and *Ptf1a* overlap completely with exocrine markers *Carboxypeptidase A1* (*Cpa1*) (A) and *Amylase* (B) (arrowheads), suggesting that the branching tips are occupied by exocrine cells at this stage.

(C) The trunk of the pancreatic branches is largely occupied by endocrine cells at E14.5, as revealed by in situ hybridization with a cocktail of endocrine precursor (E.P.) probes, including *Ngn3*, *Pax4*, *Pax6*, *NeuroD*, *Isl1*, *Brn4*, *Myt1*, *MafB*, *Arx*, *Wbscr14*, and *VDR*. No staining was observed within distal tips (arrowheads). Some trunk cells appear to express none of the E.P. markers (arrows). Asterisks indicate the early islets.

(D) No overlap was observed between the tip marker *Cpa1* (arrowheads) and the trunk maker *Ngn3* at E14.5 by double-labeled in situ hybridization.

(E–H) Section in situ hybridization of dorsal pancreata at E12.5, when pancreatic branching morphogenesis had just begun. Strong *Cpa1* expression was detected in the newly formed branches (G), whereas the expression of two other tip markers, *c-Myc* and *Ptf1a*, is quite weak at this stage (E and F). *Cpa1* and *Ngn3* again exhibit nonoverlapping expression patterns (H). Arrowheads indicate branching tips. Hollow arrowheads indicate the "cleft" region between future branches where Cpa1 is downregulated.

after E11.5, branching of the pancreas begins. By E12.5, there are well-formed primary branches, and Cpa1 expression is now largely restricted to the branching tips (Figures 3B, 3F, and 3J, arrows). The tip-restricted expression pattern of Cpa1 persists through successive branching and growth of the pancreatic tree (Figures 3C, 3D, 3G, 3H, 3K, and 3L). Note that Cpa1 protein levels vary among tip cells, perhaps reflecting their different mitotic stages.

At all embryonic stages examined, Cpa1$^+$ cells do not express mature endocrine hormones (Figures 3A–3D) or the early endocrine progenitor marker, Ngn3 (Figures 3E–3H). In contrast, all Cpa1$^+$ cells do express Pdx1 and constitute a subset of Pdx1$^+$ epithelial cells (Figures 3I–3L). Quantification demonstrates that Cpa1$^+$ tip cells represent 13.3%, 13.6%, and 16.8% of total Pdx1$^+$ cells at E12.5, E13.5, and E14.5, respectively. In addition, Cpa1$^+$ tip cells proliferate ~3–5 times faster than trunk epithelial cells, as revealed by staining with the mitotic marker phospho-histone H3 (Figures 3M, 3M′, and 3N). Thus, the Ptf1a$^+$cMyc^{High}Cpa1$^+$ tip cells represent a novel, fast-proliferating subset of the general pancreatic precursors marked by Pdx1 expression.

Genetic Lineage Tracing of the Tip Progenitors

To determine whether the tip cells constitute multipotent progenitors or committed cells, we generated a *Cpa1CreER^{T2}* mouse line that expresses an inducible form of Cre recombinase (*CreER^{T2}*) from the Cpa1 genomic locus (Figures S3) (Metzger and Chambon, 2001). By crossing this mouse line with the reporter line, *R26R*, administration of tamoxifen (TM) allows permanent marking of *Cpa1*-expressing cells at designated developmental stages and allows us to follow the fate of Cpa1 progeny through subsequent development (Figure 4A).

To validate the *Cpa1CreER^{T2}* line, we first examined whether *CreER^{T2}* expression is restricted to Cpa1$^+$ cells and whether *CreER^{T2}* activation is strictly dependent upon TM. *Cpa1CreER^{T2};R26R* animals were labeled with one TM injection at E13.5 (Figure 4C) and analyzed 20 hr later. Uninjected animals served as controls (Figure 4B). Many βgal$^+$ cells were present in the pancreas of TM-injected embryos (Figure 4C), which all coexpress Cpa1 (Figure 4C, arrowheads). In contrast, no βgal$^+$ cells were detectable in the absence of TM (Figure 4B). These data show that *CreER^{T2}* expression is restricted to Cpa1$^+$ cells and, importantly, activation of *CreER^{T2}* is strictly TM dependent. A similar experiment on adult animals confirmed that *CreER^{T2}* expression is restricted to adult exocrine acini (Figure 4E, arrowhead), but not islets (Figure 4E, arrow) or ducts. Again, *CreER^{T2}* activation is TM dependent (Figure 4D). A maximum of ~20% labeling efficiency is observed in the adult (4 mg TM × 3).

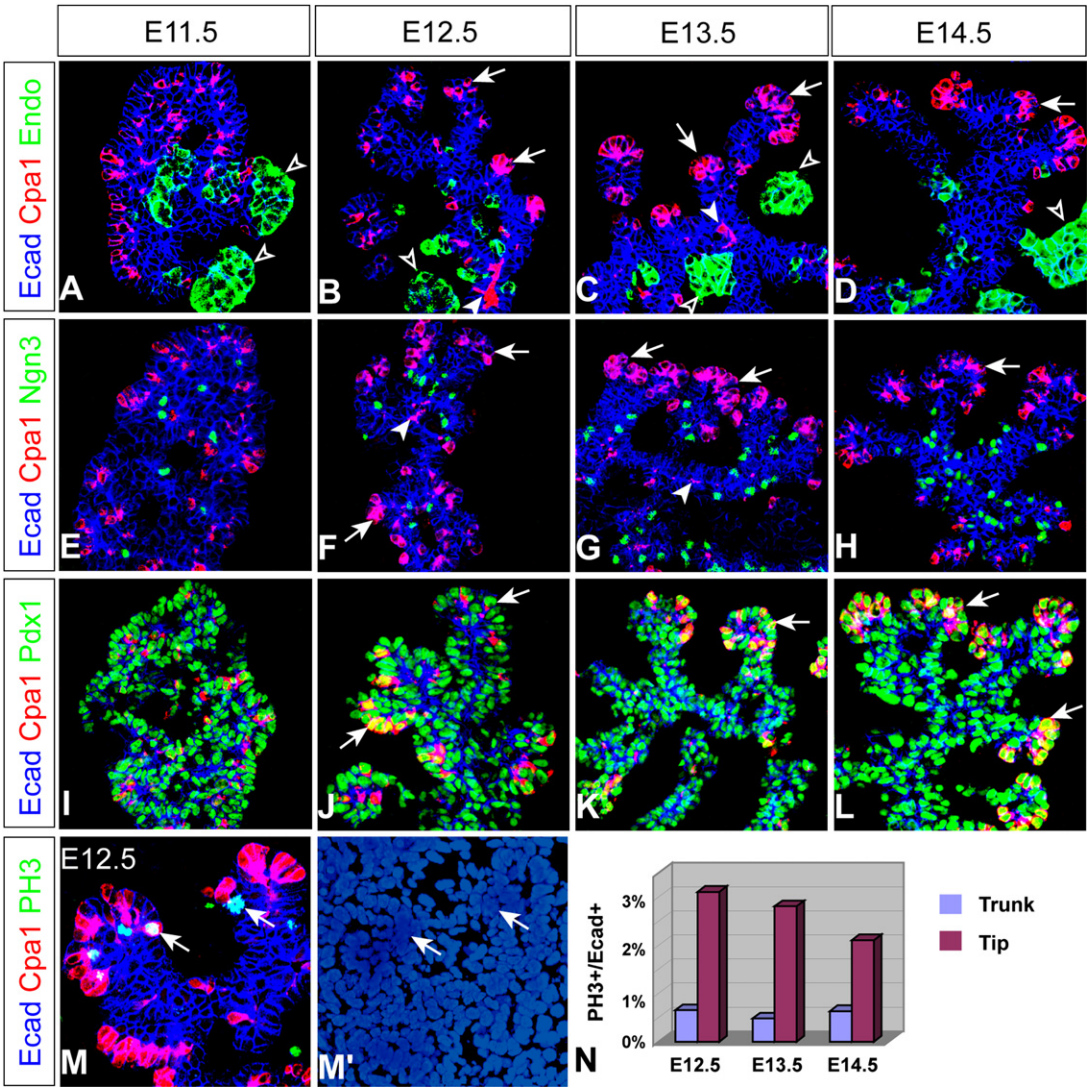

Figure 3. Cpa1 Labels a Distinct Tip Progenitor Population

Confocal immunofluorescent images of pancreatic tissues. Cpa1 expression is evident in a scattered population of cells in E11.5 pancreatic bud (A, E, and I) and restricts to the branching tips of the growing pancreatic tree starting around E12.5 (B–D, F–H, and J–L; arrows). There is no overlap between Cpa1 and endocrine hormones (A–D), or between Cpa1 and the endocrine progenitor marker, Ngn3 (E–H), at all stages examined. In contrast, all Cpa1$^+$cells co-express Pdx1 (I–L, arrows). Ecad: E-cadherin, a marker of pancreatic epithelial cells. Endo: the four major endocrine hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) were simultaneously recognized with a mixture of antibodies. Hollow arrowheads indicate early glucagon$^+$ endocrine clusters. (M) Mitotic cells in E12.5 pancreas was recognized by the G2/M phase marker phospho-Histone H3 (PH3). Arrows indicate mitotic cells. (M′) Corresponding DAPI staining of nuclei. (N) Quantitative analysis of the proliferation rate of Cpa1$^+$ versus Cpa1$^-$ pancreatic epithelial cells. Percentile was calculated as Cpa1$^+$ mitotic epithelial cells (tip, red bar) and Cpa1$^-$ mitotic epithelial cells (trunk, blue bar) against the total number of epithelial cells.

TM has been shown to induce nuclear translocation of *CreER^{T2}* within 6 hr and to remain in the nucleus for approximately 36 hr (Ahn and Joyner, 2004; Danielian et al., 1998). Consistent with these reports, TM injection at E8.0, ~36 hr before the onset of *Cpa1* expression, yielded no βgal$^+$ cells in *Cpa1CreER^{T2};R26R* embryos, whereas many βgal$^+$ cells were present if TM was given at E9.5 (data not shown), suggesting that CreER^{T2} is active from 12 to 36 hr after injection.

Note that, in addition to *CreER^{T2}*, an *IRES-H2BYFP* element was knocked into the *Cpa1* locus (Figure S2). The presence of the IRES element should allow coexpression of H2BYFP with CreER^{T2} from the Cpa1 locus. However, we only detect YFP autofluorescence in the adult, but not the embryonic pancreas of *Cpa1CreER^{T2}* animals. This is likely due to the fact that the expression level behind the IRES element is generally much reduced, and that Cpa1 expression in the embryos is weaker than that in the adults.

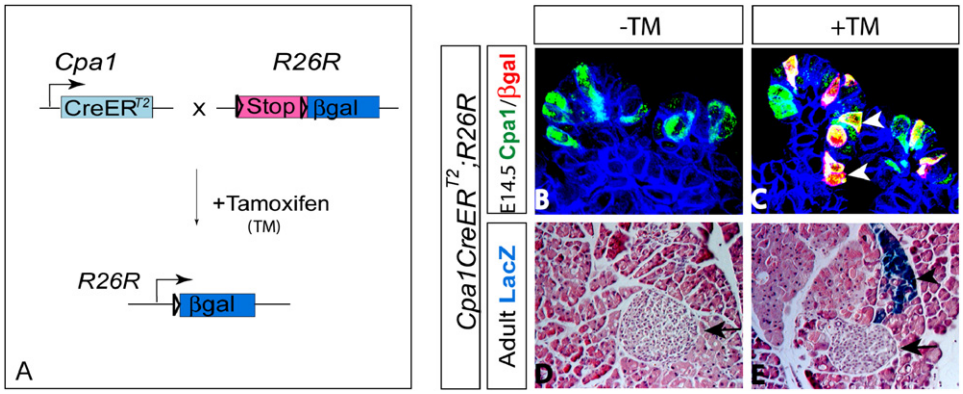

Figure 4. Strategy of Genetic Lineage Tracing with *Cpa1CreER^{T2}* Knockin Mouse Line

(A) An inducible form of Cre recombinase (CreER^{T2}) expressed specifically from the Cpa1 locus will remove the floxed stop cassette from the *R26R* allele only in the presence of TM, which allows permanent βgalactosidase (βgal) expression in the labeled cells as well as all their progeny. Triangles represent loxP sites.

(B and C) Immunofluorescence of E14.5 *Cpa1CreER^{T2};R26R* pancreata. No βgal$^+$ cells were detected in the absence of TM (B) whereas many Cpa1$^+$ βgal$^+$ cells were observed in the presence of TM (C, arrowhead).

(D and E) LacZ staining of two-month old adult *Cpa1CreER^{T2};R26R* animals. With TM injection, LacZ signal was detected in the exocrine compartment of the pancreas (E, arrowhead), but not islets (E, arrow) or ducts. In the absence of TM, no lacZ signal was detectable (D). Samples in (D) and (E) were counter-stained with Hematoxylin and Eosin.

Cpa1$^+$ Tip Progenitors are Multipotent and Give Rise to Endocrine, Exocrine, and Duct Cells before E14

Cpa1CreER^{T2};R26R embryos were labeled with a single pulse of TM at different developmental stages and analyzed at E18.5 (Figure 5 and Figures S3A). Cpa1$^+$ cells marked at E9.5, E10.5, E11.5, and E12.5 gave rise to all three major cell types of the pancreas (Figures 5A–5C, 5E–5G, 5I–5K, and data not shown)—i.e., endocrine cells (Figures 5A–5C, arrows), exocrine cells (Figures 5E–5G, hollow arrowheads), and duct cells (Figures 5E–5G, white arrowheads). Notably, TM injections at both E11.5 and E12.5 should label Cpa1$^+$ cells when they reside within the branching tips (labeling occurs around E12–E13 and E13–E14, respectively), suggesting that the early branching tip cells are multipotent progenitors. In sharp contrast, Cpa1$^+$ cells marked at E13.5 or after generate only exocrine progeny (Figures 5D, 5H, and 5L, arrowheads).

The observation that Cpa1$^+$ tip cells produce multiple pancreatic cell types before E14 does not necessarily mean that Cpa1$^+$ cells are a homogeneous population of multipotent progenitor cells. It is possible that they are composed of a mixture of separate progenitors. To distinguish between these two possibilities, we examined clones of cells that likely derive from single Cpa1$^+$ cells (Figures S4). By varying TM doses, we first determined that, at 0.5 mg/animal, only a small number of discrete clusters of cells (0∼10 clusters) are present (Figures S4D and S4E, arrows), likely representing clones. In contrast, at 2 mg/animal, around 5%–20% of the pancreatic cells are labeled (Figures S4F). No lacZ-positive cells were ever observed in the absence of TM (Figures S4A). Clones of cells labeled at E11.5 and harvested at E13.5 typically contain just a few cells (Figures S4B and S4C), whereas clones harvested at E18.5 generally contain dozens of cells (Figures S4G and S4H).

For clonal analysis, pancreata with a single lacZ$^+$ clone or two well-separated clones were selected. Individual clones were sectioned through their entirety and stained with insulin to visualize the primitive islets. The three major pancreatic cell types can be easily distinguished based on insulin and lacZ signals: endocrine cells reside within the primitive islets (Figures S4I, arrows); duct cells exhibit an elongated shape and reside within ducts (Figures S4I, black arrowheads); and exocrine cells appear as large round cells that are part of a rosette structure (Figures S4I, white arrowheads). Out of 43 clones examined, 32 (74%) are composed of exocrine, endocrine, and duct cells (Figures S4). In addition, a small number of clones were found to contain either a mixture of two cell types or only exocrine cells (Figures S4). Although the number of clones examined is rather small, these results strongly suggest that individual Cpa1$^+$ tip progenitors labeled between E12 and E13 are tripotent.

Multipotent Tip Progenitors Give Rise to Mature Endocrine Cells in a Stepwise Fashion

We next sought to visualize the various differentiation steps that lead from the Cpa1$^+$ multipotent tip progenitors to mature pancreatic cells. Among the three major pancreatic cell types, the differentiation steps of the endocrine cells are best understood (Wilson et al., 2003). The earliest recognizable endocrine progenitors are Ngn3$^+$ (Gradwohl et al., 2000; Schwitzgebel et al., 2000), which subsequently give rise to a number of late progenitor cell types expressing markers such as NeuroD, Pax4, Pax6, Arx, etc. (Wilson et al., 2003). Mature endocrine cells derive from these late progenitor cells.

To visualize each step of this differentiation process, we labeled *Cpa1CreER^{T2};R26R* embryos with a single dose of TM at E12 (labeling E12.5–E13.5) and harvested them on

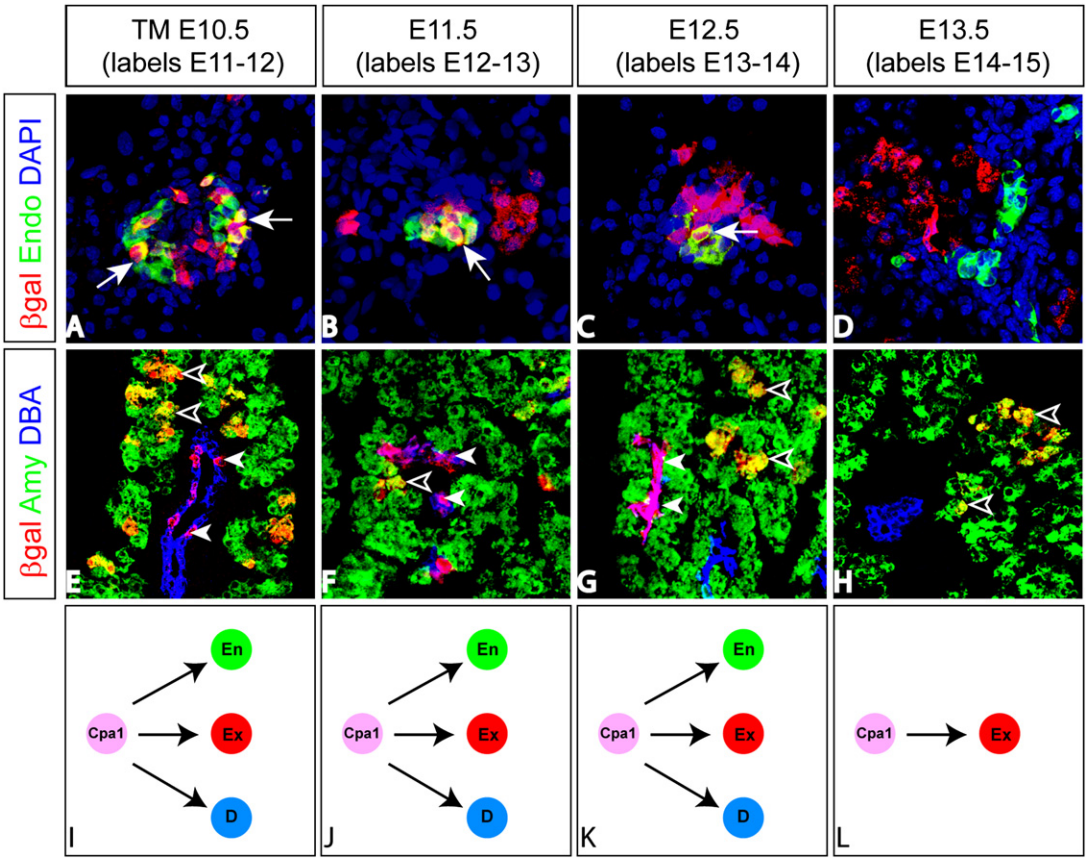

Figure 5. Cpa1$^+$ Cells Are Multipotent before E14

Cpa1CreER^{T2};R26R embryos were labeled with one dose of TM at different embryonic stages. Pancreata were harvested at E18.5. Cpa1$^+$ cells pulsed at E10.5, E11.5, and E12.5 (labeling occurs at E11–12, E12–13, and E13–14, respectively) gave rise to endocrine (A–C, arrows), exocrine (E–G, hollow arrowheads), and duct (E–G, white arrowheads) progeny. In contrast, Cpa1$^+$ cells pulsed at E13.5 (labeling E14–15) generate only exocrine (H, hollow arrowheads), but not endocrine (D) or duct (H) cells. Endo, four major endocrine hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) were simultaneously recognized with a mixture of antibodies; Amy, Amylase, a mature exocrine marker; DBA, a pancreatic duct marker. (I–L) Summary of the lineage of Cpa1$^+$ cells at different embryonic stages. En, endocrine; Ex, exocrine; D, duct.

three successive days for analysis (Figure 6 and Figures S3B). One day after TM injection, the initial population of TM-responsive cells expresses both βgal and Cpa1 as expected (Figure 6A, arrows). Consistent with the labeling of an early multipotent progenitor cell type, these cells were negative for the early endocrine marker, Ngn3 (Figure 6D), the late endocrine marker, Pax6 (Figure 6G), and all endocrine hormones (Figure 6J). Starting from 2 days after labeling, we observed that Cpa1$^-$ βgal$^+$ cells appear in the trunk region of the branches (Figures 6B and 6C, arrowheads) in addition to Cpa1$^+$βgal$^+$ cells residing in the tips (Figures 6B and 6C, arrows). This observation, together with the data that most Cpa1$^+$ cells are multipotent before E14 (Figure 5 and Figures S4), suggests that some Cpa1$^+$ are capable of limited self-renewal (i.e., producing more Cpa1$^+$ multipotent tip cells as well as Cpa1$^-$ differentiated progenies).

Starting from E14, some progeny of Cpa1$^+$ cells labeled at E12 begin to express Ngn3 (Figures 6E and 6F, arrowheads), indicating that they have become committed endocrine progenitors. One day later, at E15, Pax6$^+$βgal$^+$ late endocrine progenitors, as well as endo$^+$βgal$^+$ mature endocrine progenies, were detected (Figures 6I and 6L). These data suggest that Cpa1$^+$ cells appear to self-renew (making more Cpa1$^+$ cells) and lay down daughters that go through a series of differentiation steps to generate mature pancreatic cells in vivo (Figure 6M).

DISCUSSION

Formation of the mammalian pancreas as a complex three-dimensional organ requires timely generation, migration, and differentiation of different cell types from multipotent progenitors. We performed genome-wide TF expression analysis of the developing pancreas and discovered multiple distinct gene expression domains that indicate the presence of specific pancreatic progenitor domains. Using a combination of serial immunofluorescence and genetic lineage tracing experiments, we propose that one type of multipotent pancreatic progenitor can be recognized by a combination of markers (Pdx1$^+$Ptf1a$^+$cMyc^{High}Cpa1$^+$), resides specifically at the

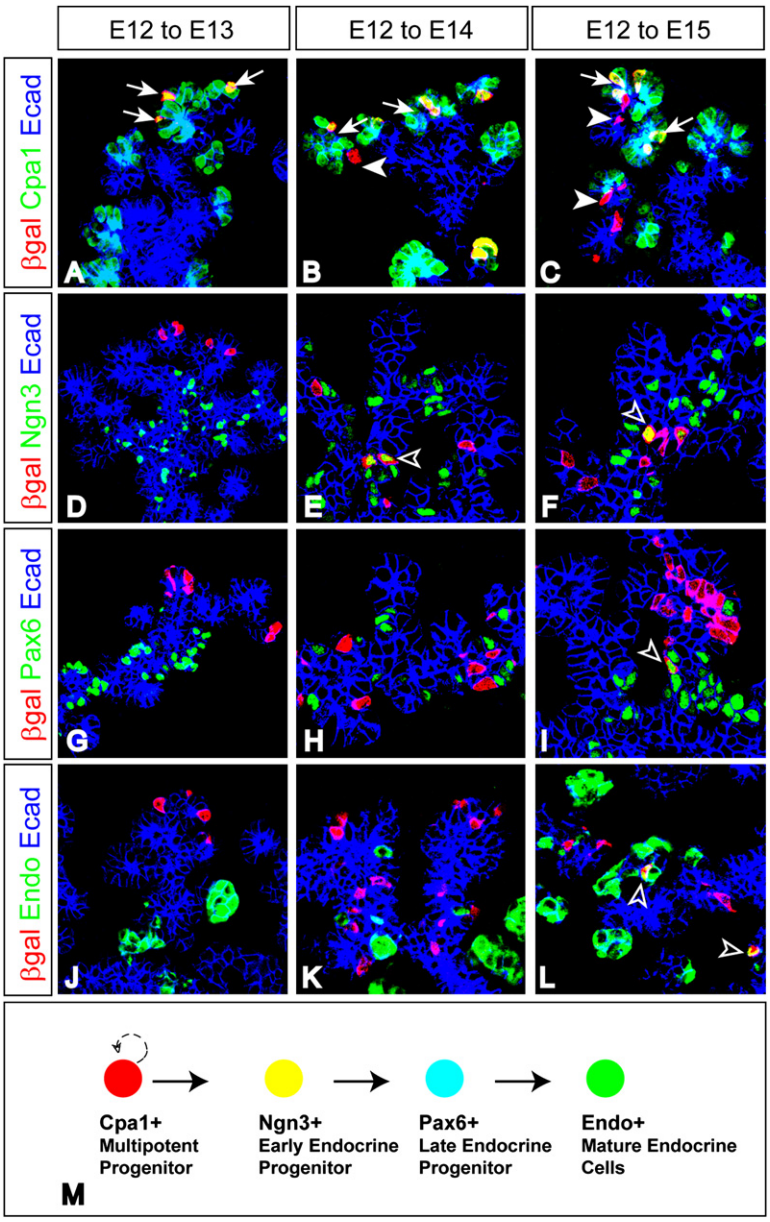

Figure 6. Stepwise Differentiation of Cpa1$^+$ Multipotent Progenitors to Mature Endocrine Cells

Cpa1CreER^{T2};R26R embryos were labeled with one dose of TM at E12 and harvested 1–3 days after injection to follow the fate of the labeled cells.

(A–C) One day after pulse labeling, all βgal$^+$ cells coexpress Cpa1 (A, arrow). Starting from 2 days after labeling (B and C), while some βgal$^+$ cells maintain Cpa1 expression and reside in the branching tips (B and C, arrows), others lose Cpa1 expression and appear in the trunk region (B and C, arrowheads).

(D–L) Cpa1$^+$ cells labeled at E12 gave rise to Ngn3$^+$ early endocrine progenitors starting from 2 days after TM (E and F, hollow arrowheads), and Pax6$^+$ late progenitors at 3 days post TM (I, hollow arrowheads). Hormone-producing mature endocrine cells also appear 3 days after TM (L, hollow arrowheads). Endo, all four major endocrine hormones (insulin, glucagons, somatostatin and PP) were detected with a mixture of antibodies.

(M) Summary of the E12 pulse-chase experiment.

branching tips of the growing pancreatic tree, and that its proliferation and differentiation play major roles in the branching morphogenesis of the pancreas. In addition, our analysis of TFs has uncovered pancreatic genes that are likely to play important roles in pancreas development and disease.

Domain Organization of the Developing Pancreas

Our data show that regionalized gene expression patterns are created very early and are maintained through successive branching and growth of the pancreatic tree. The embryonic pancreas contains relatively few gene expression domains, in contrast to the developing nervous system and kidney, where many more expression patterns have been discovered in similar screen efforts (Gray et al., 2004; A.P. McMahon, personal communication). Given the large number of cell types that exist in the adult brain and kidney, the simple domain organization of the developing pancreas may reflect the relatively small number of cell types that are produced in the developing pancreas. What causes the tip and trunk of the pancreatic branches to have different gene expression patterns? In other developing organ systems, such as the lung, nervous system, and limb bud, focal sources of morphogens are responsible for creating different domains of gene expression in an otherwise homogeneous tissue (Hogan, 1999; Jessell, 2000). Such focal sources of morphogens have not been identified in the developing pancreas. In fact, although many signaling molecules are expressed in the developing pancreas, few exhibit regionalized expression. One notable exception is the Notch pathway. Notch receptors and Hes1, an effector of Notch pathway, have elevated

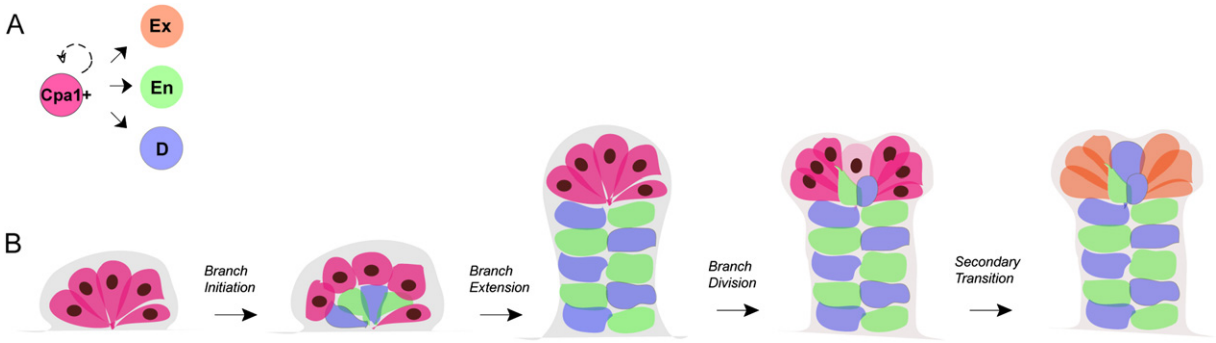

Figure 7. Multipotent Progenitors Guide Pancreatic Organogenesis

(A) Cpa1$^+$ multipotent progenitors give rise to exocrine, endocrine, and duct cells in vivo and may undergo limited self-renewal.

(B) Early pancreatic buds are composed primarily of multipotent progenitors. At the onset of branching morphogenesis (~E12), the multipotent cells may divide asymmetrically such that they are propelled away from the center of the pancreatic buds, thus producing branches. Continued fast proliferation and differentiation of these progenitors into endocrine and duct cells generate the trunk of the branches. When the branching tip divides, Cpa1 downregulates in the cleft region. Around E14, Cpa1$^+$ tip cells restrict to exocrine fate during the secondary transition.

expression in distal tips of the embryonic pancreatic endoderm (Apelqvist et al., 1999; Wang et al., 2005). Similarly to other developing organ systems, the Notch pathway functions in pancreas to suppress differentiation and maintain progenitors in an undifferentiated state (Apelqvist et al., 1999; Esni et al., 2004; Hald et al., 2003; Jensen et al., 2000; Murtaugh et al., 2003). These studies suggest that tip cells of the branching pancreas are actively maintained in a progenitor state by elevated Notch signaling. It is unclear, however, as to what causes the preferential activation of Notch pathway in the early distal tips. Although Fgf10 signaling has been reported to promote or maintain Notch expression in embryonic pancreas (Hart et al., 2003; Norgaard et al., 2003), pancreatic expression of Fgf10 is diffuse and rather transient (Bhushan et al., 2001).

In other tissue and organ systems, it has been shown that stem cells reside in specific "niches," or microenvironments (Fuchs et al., 2004). Similarly, the multipotent tip progenitors of the embryonic pancreas may also have a supporting niche, suggesting that mesenchymal cells surrounding the branching tips may exhibit different properties. However, no differential gene expression has been observed in the developing pancreatic mesenchyme.

Multipotent Progenitors of the Pancreas

Our genetic lineage tracing studies show that one type of multipotent pancreatic cell is localized specifically to the branching tips. Beside the tip cells, most of the trunk cells appear to be committed endocrine progenitors (Figure 2C). In addition, there is a population of trunk epithelial cells that do not express any of the known endocrine progenitor markers (Figure 2C). Since the trunk of the developing pancreatic tree is the predecessor of the adult duct system, we favor the idea that these unidentified cells represent duct progenitors. Nevertheless, we cannot rule out the possibility that some of these Cpa1$^-$ cells are also multipotent. Further genetic studies with additional molecular markers should help resolve this important issue.

Our lineage analysis is a test of developmental fate, but not potential of the Cpa1$^+$ cells. It remains possible that the Cpa1$^+$ cells are competent to produce endocrine/duct cells beyond E14, but the inducing cues are absent. However, recent observations from our laboratory suggest that transplanting dissociated E16.5 or E17.5 pancreatic cells into cultured E10.5 pancreatic buds fail to produce clones with multilineage contribution. In contrast, similar experiments with cells collected before E14 resulted in multilineage clones (R.I. Sherwood and D.A.M., unpublished data). These observations support our conclusion that multipotent pancreatic cells disappear after E14.0, although it is formally possible that a small number may persist into later development.

Genetic studies have shown that, during adulthood, beta cells do not come from multipotent progenitors (Dor et al., 2004; Brennand et al., 2007; Teta et al., 2007). Furthermore, pancreatic organ size appears to be constrained by the initial progenitor population, and there is little cellular compensation (Stanger et al., 2007). These findings, together with the results of our analysis, suggest that Cpa1$^+$ cells before E14 may represent the only multipotent pancreatic progenitors.

Integrating Pancreatic Branching and Cell Type Specification

Our data suggest a simple model of how pancreatic branching is integrated with cell type specification. The early pancreatic bud consists primarily of multipotent progenitors (Figure 7A). When branching begins, Pdx1$^+$Ptf1a$^+$cMyc^{High}Cpa1$^+$ multipotent cells divide rapidly, and possibly in a directional fashion, such that they are propelled outward from the epithelial plane, thus initiating branches. The proliferation and outgrowth of the multipotent tip cells leave behind their more differentiated progeny (i.e., the endocrine and duct cells), which make up the trunk of the branches (Figure 7B). Eventually, endocrine cells differentiate and migrate out of the epithelium, leaving the trunk to be made entirely of duct cells. At

midgestation during the so-called "secondary transition" (Pictet and Rutter, 1972), tip progenitors undergo a developmental switch that converts them into exocrine cells (Figure 7B).

Pancreatic branching continues after the secondary transition. The mechanism for continued branching, however, remains unknown. In addition, we have observed that the timing of conversion from multipotent to acinar fate is not synchronized, with Amylase expression appearing in scattered tip cells from E13.5. Reduced production of endocrine and duct cells at these later stages may explain why an earlier study has suggested that duct cells are only specified before E12.5 (Gu et al., 2002). The methodology we used to detect duct cells (whole-mount X-gal staining of E18.5 pancreas) may be more sensitive than that employed in the previous study (section staining of alkaline phosphatase [AP] on 2 month adult pancreas).

Many mammalian organs, including the lung, kidney, mammary gland, and prostate gland, develop via branching morphogenesis (Davies, 2002; Hogan and Kolodziej, 2002). The branching tips of many of these organs express regionalized markers and exhibit increased proliferation (Fisher et al., 2001; Mollard and Dziadek, 1998; Xue et al., 2001). A recent study of developing kidney provided evidence that tip cells of the ureteric bud are bipotential and contribute to both tips and trunks (Shakya et al., 2005). Together with our analysis, these studies suggest that it may be a common theme for all branching organs to locate their stem/multipotent cells at tips.

Cpa1 as a Multipotent Progenitor Marker

The current consensus view is that Pdx1 marks multipotent pancreatic progenitor cells. Although this is likely to be the case at the pancreatic bud stage, Pdx1 continues to be expressed broadly throughout early embryogenesis, and cannot be used to distinguish between different progenitor pools. Cpa1, on the contrary, marks only a subset of $Pdx1^+$ cells and these cells are multipotent in vivo before E14. Although our data show that these multipotent tip cells are positive for Pdx1, Ptf1a, c-Myc, and Cpa1 and negative for differentiated lineage markers, we propose that a simpler combination of markers ($Pdx1^+Cpa1^+Amylase^-$) is sufficient to unambiguously identify multipotent pancreatic progenitor cells in vivo and perhaps in vitro as well.

It is noteworthy that the expression pattern of *Cpa1* is highly reminiscent of that of the pancreatic TF *Ptf1a* (Krapp et al., 1996). Expression of *Ptf1* first appears around E10 and later restricts to exocrine tissues (Krapp et al., 1996). Due to weak expression of *Ptf1a* before E12.5, we were not able to assess whether *Cpa1* and *Ptf1a* are always coexpressed in individual cells before E12.5. Nevertheless, it is likely that Ptf1a controls Cpa1 expression. The mutant phenotype of *Ptf1a* suggests that it plays a critical role in specifying the early pancreatic multipotent progenitors (Kawaguchi et al., 2002). This is consistent with the idea that Cpa1 marks multipotent cells.

The ability to identify multipotent pancreatic progenitors has practical implications. Islet transplantation has recently been shown to be an effective method to treat type I diabetes patients (Lakey et al., 2006). To generate large amounts of beta cells for transplants, many ongoing efforts are focused on the directed differentiation of beta cells from embryonic stem cells. As an important intermediate cell type in these cultures, it is critical to recognize the early multipotent pancreatic precursors so that their survival and in vitro expansion can be optimized. Additionally, it would also be interesting to see whether such $Pdx1^+Cpa1^+Amylase^-$ cells reappear in the adult pancreas after injury and serve as adult stem cells for tissue regeneration.

EXPERIMENTAL PROCEDURES

In Situ Hybridization

Whole-mount in situ hybridization screen with the TF library on E9.5 mouse embryos and E14.5 pancreata was performed essentially as described previously (Gray et al., 2004). Briefly, a plasmid library that represents ~1,100 independent mouse TFs served as template for PCR amplification of the inserts. Digoxigenin-labeled cRNA probes were made directly from the PCR products using T7, T3, or Sp6 polymerases (Roche) and purified through Micro Bio-spin columns (Bio-Rad). E9.5 embryos and E14.5 pancreata were dissected and fixed with 4% paraformaldehyde overnight. After proteinase K treatment, the pancreata were hybridized with individual cRNA probes. Posthybridization washes and antibody incubation were performed with a BioLane in situ hybridization machine (Holle and Huttner, AG). Signals were developed with BM purple (Roche). Samples were cleared in 80% glycerol and photographed.

Single- and double-labeled section in situ hybridization on paraffin-embedded tissues was performed as previously described (Gray et al., 2004). For double-labeled in situ hybridization, two different probes were labeled with either digoxigenin or fluorescein. The first probe was detected with AP-conjugated anti-digoxigenin antibody, and developed with NBT/BCIP (Biorad), which yields a purple precipitate. After the NBT/BCIP reaction, the AP-conjugated anti-digoxigenin antibody was inactivated at 85°C and the slides were subsequently incubated with AP-conjugated anti-fluorescin antibody and detected with INT/BCIP (Roche), which yields a reddish brown precipitate.

Immunohistochemistry

Mouse embryos and pancreata were fixed by immersion in 4% paraformaldehyde from 1 to 6 hr depending on the age. Samples were subsequently incubated in 30% sucrose solution overnight and embedded with OCT compound (Vector). The following primary antibodies were used: rat anti-E-cadherin (Zymed), rabbit anti-carboxypeptidase A1 (Biogenesis), goat anti-Ngn3 (Santa Cruz), Guinea pig anti-insulin (Dako), guinea pig anti-glucagon (Linco), guinea pig anti-pancreatic polypeptide (Linco), goat anti-somatostatin (Santa Cruz), goat anti-Pdx1 (Santa Cruz), guinea pig anti-Pdx1 (gift of Dr. Chris Wright), rabbit anti-βgalactosidase (Cappel), goat anti-βgalactosidase (Biogenesis), rabbit anti-Amylase (Sigma), sheep anti-Amylase (Abcam), mouse anti-Ki67 (BD PharMingen), rabbit anti-Pax6 (Chemicon), and FITC-conjugated mouse anti-phospho-histone H3 (Upstate). Rodamin-red-X, FITC, Cy5, and Alexa dye-conjugated donkey secondary antibodies were obtained from Jackson Immunoresearch Laboratories and Molecular Probes Inc. Biotin-labeled *Dolichos biflorus* agglutinin is from Vector Laboratories Inc.

Generation of *Cpa1CreER^{T2}* Knockin Mice

The knockin vector for *Carboxypeptidase A1* (*Cpa1*) was generated by inserting a *CreER^{T2}-IRES-H2BYFP-ACN* cassette between a 2.7 kb 5′ arm and a 4.5 kb 3′ arm. Both arms were derived from PCR amplification of AV3 ES cell genomic DNA and confirmed by sequencing. ACN is a neomycin selection cassette that self-deletes during germ line

transmission (Bunting et al., 1999). The linearized construct was electroporated into AV3 ES cells. After neomycin selection, positive clones were picked and screened by Southern blot with both a 5′ probe and a 3′ probe. Three recombined ES clones were injected into C57BL/6 blastocysts. Two gave germ line transmission. PCR analysis confirmed that the ACN selection cassette was self-deleted from all germ line-transmitted animals. Since *Cpa1CreER*$^{T2-/-}$ homozygous animals are healthy and fertile, this mouse line was maintained as homozygotes. The Rosa-loxP-stop-loxP-lacZ reporter mice (*R26R*) were purchased from the Jackson Laboratory and maintained as homozygotes. All animal experiments described in this article have been approved by Harvard University's Institutional Animal Care and Use Committee.

Genetic Lineage Tracing

TM (Sigma T-5648) was dissolved in corn oil (Sigma C-8267) at 10 mg/ml. *Cpa1CreER*^{T2} homozygous males were mated with *R26R* homozygous females to produce double-heterozygous *Cpa1CreER*^{T2};*R26R* embryos. The noon of the day of a vaginal plug was designated as E0.5. We have found that TM doses over 2 mg per pregnant female (weighing ~30–40 g each) induced significant embryonic lethality. All embryonic experiments were performed with less than 2 mg TM per animal given intraperitoneally.

Supplemental Data

Supplemental Data, including five additional figures and one table, are available online at http://www.developmentalcell.com/cgi/content/full/13/1/103/DC1/.

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